1-Methyl-4-phenylpyridinium affects fast axonal transport by activation of caspase and protein kinase C

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Parkinson's disease (PD), a late-onset condition characterized by dysfunction and loss of dopaminergic neurons in the substantia nigra, has both sporadic and neurotoxic forms. Neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its metabolite 1-methyl-4-phenylpyridinium (MPP+) induce PD symptoms and recapitulate major pathological hallmarks of PD in human and animal models. Both sporadic and MPP+-induced forms of PD proceed through a ''dying-back'' pattern of neuronal degeneration in affected neurons, characterized by early loss of synaptic terminals and axonopathy. However, axonal and synaptic-specific effects of MPP+ are poorly understood. Using isolated squid axoplasm, we show that MPP+ produces significant alterations in fast axonal transport (FAT) through activation of a caspase and a previously undescribed protein kinase C (PKCδ) isoform. Specifically, MPP+ increased cytoplasmic dynein-dependent retrograde FAT and reduced kinesin-1-mediated anterograde FAT. Significantly, MPP+ effects were independent of both nuclear activities and ATP production. Consistent with its effects on FAT, MPP+ injection in presynaptic domains led to a dramatic reduction in the number of membranous profiles. Changes in availability of synaptic and neurotrophin-signaling components represent axonal and synaptic-specific effects of MPP+ that would produce a dying-back pathology. Our results identify a critical neuronal process affected by MPP+ and suggest that alterations in vesicle trafficking represent a primary event in PD pathogenesis. We propose that PD and other neurodegenerative diseases exhibiting dying-back neuropathology represent a previously undescribed category of neurological diseases characterized by dysfunction of vesicle transport and associated with the loss of synaptic function.

cytoplasmic dynein | kinesin | Parkinson's disease | synaptic vesicle | caspase

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease with 1% of people >50 and 5% of those >85 developing PD (1). First described in 1817, PD is associated with a dying back of axons projecting from substantia nigra pars compacta (SNpC) to the striatum. When >80% of striatal dopaminergic synapses from nigral neurons no longer function, a shortage of dopamine in the striatum causes the movement defects that characterize PD (2). Although the proximate cause of PD is well understood, molecular pathogenesis in PD remained unknown. Approximately 95% of PD cases are sporadic, with an undetermined fraction resulting from environmental factors like 1-methyl-4-phenylpyridinium (MPTP) and other toxins (3). Ideally, treatments that prevent loss of affected neurons and maintain neuronal function will be developed, but this requires a better understanding of underlying molecular pathogenesis in PD (2).

PD is unique among adult-onset neurodegenerative diseases in the existence of toxin-mediated forms of the disease that mirror the neuropathology observed in sporadic PD. MPTP and its metabolite 1-methyl-4-phenylpyridinium (MPP+) is the best-characterized example of a toxin-induced PD (4), although others such as paraquat and rotenone have been documented (3). MPP+ generally is thought to target mitochondria and generate reactive oxygen species, which directly or indirectly lead to an apoptotic response, as characterized by activation of caspases (5, 6). The relationship between sporadic and toxin-based mechanisms for PD is unknown, but similarities in symptoms with human PD have made the MPTP model a powerful tool for exploring neuropathology in PD (2, 4).

Although PD neuropathology is characterized largely by loss of dopaminergic neuron function, neuronal degeneration in PD extends beyond dopaminergic systems, and not all dopaminergic neurons are similarly affected (7). Some cell types are largely unaffected in PD. For example, MPTP toxicity does not affect viability of glia, where most MPTP is metabolized to produce the toxic MPP+ metabolite (8). Similarly, the highest cellular levels of MPP+ are in the adrenal medulla, but there is no loss of chromaffin cells (9). These observations suggest that one or more cellular processes critical for appropriate neuronal function is altered selectively in PD.

The lack of protein synthesis in axons and complex functional architecture of neurons render neuronal cells extremely dependent on fast axonal transport (FAT) mechanisms (10). After synthesis and packaging in the neuronal cell body, membrane proteins needed for proper axonal and synaptic function must be transported to their final destination in axons and terminals. Maintenance of axons connectivity also requires that signaling endosomes containing activated neurotrophin receptors and other signaling components are transported retrogradely from synaptic terminals and axons to the neuronal cell body (11). Thus, basic neuronal functions including growth, information processing, and trophic factor-dependent survival all rely on appropriate FAT. Kinesin-1 and cytoplasmic dynein (CDyn) are the major microtubule (MT)-based molecular motors responsible for anterograde and retrograde FAT, respectively (10). The importance of FAT on neuronal function and survival is demonstrated by the fact that partial loss-of-function mutations in either kinesin-1 (12) or CDyn subunits (13, 14) result in late-onset degeneration of specific neuronal populations. Significantly, neuropathologies associated with mutations in molecular motors display a "dying-back" pattern of neurodegeneration (15, 16) and adult onset of symptoms (17). Thus, alterations in either anterograde or retrograde FAT can suffice to produce a dying-back neuropathy (16, 18, 19).

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Abbreviations: CDyn, cytoplasmic dynein; FAT, fast axonal transport; MPP+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine; MT, microtubule; PD, Parkinson's disease; PKCδS, PKCδ peptide substrate.

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No consensus exists on the sequence of events that lead to neuronal dysfunction in PD, but neuropathological studies of sporadic PD brains indicate that loss of synaptic terminals in the striatum precedes loss of dopaminergic neurons in the substantia nigra pars compacta (20). This hallmark of PD also is seen in MPTP-treated mice (21) and monkeys (22, 23). In addition, ultrastructural observations suggest early axonal pathology in PD patients (24, 25). These observations suggest that alterations in FAT could represent an important pathogenic event in PD.

Although a dying-back axonopathy is a hallmark of PD, axon-specific effects of MPP+ have not been addressed. In this study, we report previously undescribed axon- and synapse-specific effects of MPP+. Isolated squid axoplasm experiments indicate that MPP+ treatment induces profound changes in FAT. Specifically, MPP+ increased CDyn-dependent retrograde FAT and reduced kinesin-1-dependent anterograde FAT. MPP+ effects were independent of ATP levels and transcription/translation. Pharmacological and biochemical experiments indicate that MPP+ effects on vesicle motility are mediated by endogenous axonal caspases and the novel protein kinase C isoform, PKCδ. Consistent with its effects on FAT, MPP+ injection at presynaptic terminals of squid giant axons resulted in a dramatic reduction in the number of synaptic vesicles. Our results provide a target for MPP+-induced toxicity and suggest that misregulation of MT motor-dependent vesicular motility may represent a key step in the pathogenesis of PD and other neurodegenerative conditions.

Results

MPP+ Affects Microtubule Motor-Based Vesicle Motility in Axons. Effects of MPP+ were evaluated in isolated squid axoplasm, a unique experimental system for evaluating axon-specific effects and pathogenic mechanisms associated with FAT (26) in isolation from neuronal cell body functions. Isolated squid axoplasm was instrumental in the original discovery of kinesin-1 (27), regulatory pathways for FAT (28), axon-specific phosphorylation events (29), and pathogenic mechanisms for neurodegenerative diseases (30, 31). Bidirectional movement of various membrane-bound organelles can be observed directly in extruded axoplasm for hours after removal of plasma membrane, with properties unchanged from intact axons (26). Video-enhanced microscopic techniques allow for real-time quantitative analysis of membrane-bound organelle movement in FAT. Typical kinesin-1-dependent transport rates are 1.5–2.0 μm/s, whereas retrograde, CDyn-dependent rates are 1–1.3 μm/s (Fig. 1*A*). These rates are maintained with little (<10%) or no reduction for >1 h after perfusion with control buffer. The lack of permeability barriers allows perfusion of axoplasm with effector molecules at known concentrations (26). Perfusion of MPP+ at a concentration of 50 μM significantly increased CDyn-dependent retrograde FAT rates, and slightly reduced kinesin-1-based anterograde FAT (Fig. 1*B*). No obvious changes in overall axoplasmic organization or peripheral MTs were noted with MPP+ perfusion (data not shown).

Although MPP+ can compromise mitochondrial function and ATP production by directly inhibiting complex I (32), mitochondrial uncoupling agents like 2,5-dinitrophenol or carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone had little effect on FAT as long as a suitable exogenous source of ATP was available (26). In this study, effects of MPP+ on FAT were independent of endogenous ATP, because 5 mM ATP was provided in the perfusion buffer. Further, increased CDyn-based membrane-bound organelle motility is inconsistent with reductions in axoplasmic ATP levels or effects on MT integrity. These results indicated that MPP+ activates CDyn-dependent vesicle motility in a manner independent of both nuclear activity and ATP availability.

PKC Activity Mediates MPP+-Induced FAT Alterations. MPP+ induced significant changes in FAT, but the molecular basis mediating these effects remained unknown. In a prior screen of kinase

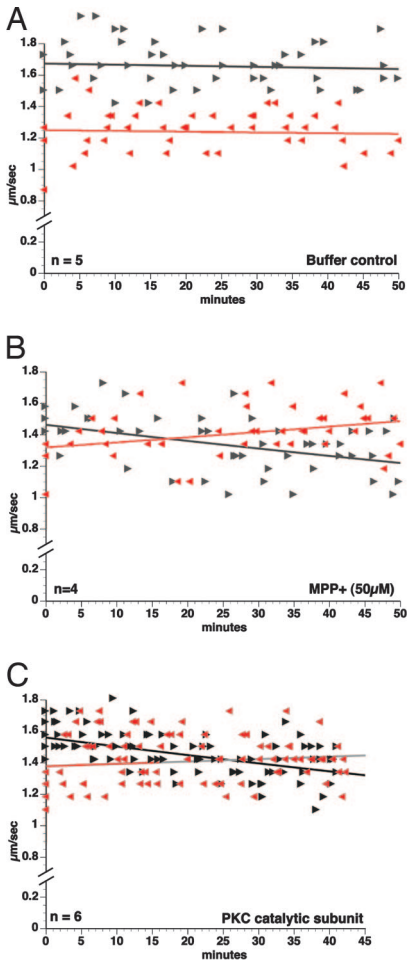

Fig. 1. MPP+ and PKC activate retrograde axonal transport and inhibit anterograde axonal transport. (*A*) FAT was analyzed in axoplasm perfused with control X/2 buffer. Under these conditions, FAT remains constant in both anterograde and retrograde directions for 90 min or more. Axoplasms were routinely analyzed for 50 min. (*B*) Perfusion of axoplasm with buffer X/2 containing 50 μM MPP+ dramatically altered both anterograde and retrograde FAT. Retrograde transport was rapidly increased, and it continued to increase over the course of the experiment. Both number retrograde moving organelles and average retrograde rates increased. In contrast, the number and average rate of anterograde moving organelles declined steadily over the course of the experiment. (*C*) Perfusion of axoplasms with the constitutively actively catalytic domain of conventional PKC significantly affects both retrograde FAT and anterograde FAT. As with MPP+, the number and average rate of retrograde moving organelles increased, and anterograde FAT declined concurrently. This suggested that effects of MPP+ might be mediated by activation of a PKC kinase activity in axoplasm. Black symbols are for anterograde and red are for retrograde.

effects on FAT, perfusion of active protein kinase C (PKC) catalytic subunits into axoplasm elicited effects qualitatively similar to induced by MPP+ (33). Perfusion of PKC catalytic subunit (Fig. 1*C*) into axoplasm increased CDyn-mediated vesicle motility while reducing kinesin-1-based vesicle motility. To determine whether PKC activity mediated MPP+ effects on FAT, we coperfused MPP+ with multiple PKC inhibitors with different modes of action (Fig. 2). Coperfusion experiments have been successful in the identification of kinase activities mediating effects of neuropathogenic proteins on FAT (30). MPP+ was coperfused with either Gö6983 (Fig. 2*A*) or Gö6976 (Fig. 2*B*). Gö6983 and Gö6976 are well characterized bisindolylmaleimide-derived pharmacological inhibitors of PKC with slightly different specificities (34). Coperfusion of either Gö6983 (Fig. 2*A*) or Gö6976 (Fig. 2*B*) at a concentration of 500 nM blocked effects of MPP+ on FAT. MPP+

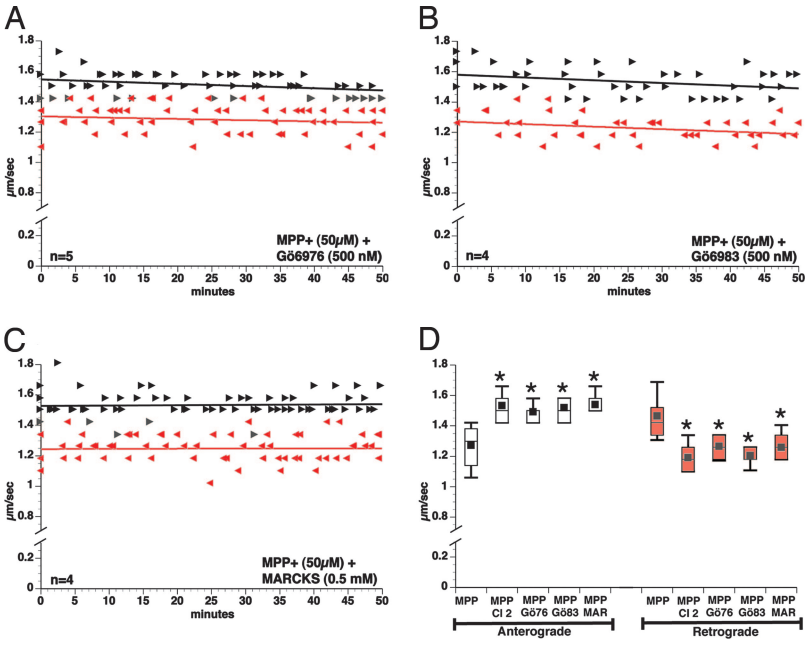

Fig. 2. Inhibitors of PKC isoforms block the effects of MPP+ on FAT. (*A* and *B*) The effects of MPP+ on FAT are completely blocked by Gö6976 (*A*) and Gö6983 (*B*), two highly specific inhibitors of both novel and conventional PKC isoforms. (*C*) Similarly, perfusion of excess (0.1 mM) MARCKS peptide, a preferred substrate for PKCs, also blocks MPP+ effects on FAT. (*D*) Effects of MPP+ on both anterograde FAT and retrograde FAT are blocked by three different PKC inhibitors Gö6976 (Gö76), Gö6983 (Gö83), and MARCKS (MAR) as well as a caspase-3 inhibitor (CI 2). Differences from MPP+ were significant at $P \geq 0.01$. Coperfusion of any of these inhibitors with MPP+-restored FAT to levels that were indistinguishable from buffer controls.

effects on FAT also were blocked by coperfusion with myristoylated alanine-rich protein kinase C substrate (MARCKS) peptide (Fig. 2*C*). MARCKS peptide is a PKC peptide substrate derived from residues 154–165 of MARCKS (35). MARCKS peptide contains the first two of four serine residues in the phosphorylation substrate domain of MARCKS and is phosphorylated at serines 5 and 9 *in vivo* and *in vitro* by PKC isoforms with very high affinity (36, 37). MARCKS was effective when coperfused with MPP+ at a concentration of 100 μM, suggesting it acted as a competitive inhibitor of PKC under these conditions. Thus, three PKC inhibitors with different modes of action were equally effective in preventing MPP+ effects on FAT (Fig. 2*D*).

Biochemical assays of PKC in axoplasm confirmed coperfusion assays. Kinase assays were designed to measure endogenous PKC activity in axoplasms by using MARCKS peptide as acceptor substrate (Fig. 3). Phosphorylation of MARCKS peptide in these assays was inhibited strongly by Gö6976, and greatly enhanced by addition of phorbol-12-myristate-13-acetate, a potent activator of PKCs (data not shown). This data indicated MARCKS peptide is phosphorylated specifically by endogenous PKCs in axoplasm. Kinase assays showed that MPP+-treated axoplasms exhibit increased PKC activation, relative to untreated controls (Fig. 3). These data indicated that axonal PKCs mediate MPP+ effects on FAT.

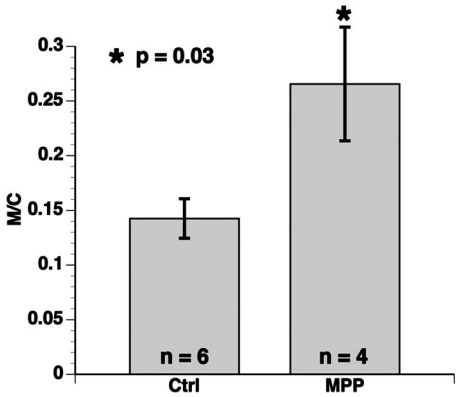

Fig. 3. MPP+ treatment increases PKC activity in axoplasm. Axoplasms treated with and without MPP+ (200 μM) were incubated in buffer X/2 containing [γ-^{32}P]ATP and either CREBpp (phosphorylation standard) or MARCKS (PKC activity) peptides. CREBpp peptide is not a substrate for PKC and serves to correct for differences in basal kinase activity and axoplasmic volumes. The MARCKS/CREBpp ratio (M/C) was significantly increased by MPP+ treatment ($P = 0.03$ in pooled *t* test).

Caspase Activity Mediates MPP+-Induced Alterations in FAT. A large body of experimental evidence suggests that activation of caspases is associated with neurotoxic effects of MPP+ *in vivo* (5, 38). To determine whether caspase activity was required for MPP+ effects on FAT, we coperfused MPP+ and Z-DEVD-fmk (Fig. 4). Z-DEVD-fmk is a potent, irreversible inhibitor of several caspases (39, 40). Coperfusion of Z-DEVD-fmk peptide prevented MPP+ effects on FAT (Figs. 2*D* and 4*A*), indicating that MPP+ effects on FAT require the proteolytic activity of endogenous axonal caspases. Perfusion of recombinant, active caspase-3 (64 nM) in axoplasm mimicked effects of MPP+ on FAT (Fig. 4*B*). Significantly, MPP+ has been shown to induce caspase 3-dependent proteolytic cleavage and activation of a specific PKC isoform *in vivo* (41), suggesting that MPP+-induced caspase activation preceded the increase in PKC activity and inhibition of FAT.

PKCδ Mediates MPP+-Induced Effects on FAT. These results suggested that activity of both caspases and PKCs mediated effects of MPP+ on FAT. However, the PKC kinase family consists of 12 or more serine/threonine-specific isoenzymes (42), and the identity of PKC isoforms mediating MPP+ effects remained to be determined. The conventional PKCs (α, β, and γ) could be ruled out because increased Ca^{2+} and DAG do not affect FAT (43). Among the novel and atypical PKCs, PKCδ is a well established substrate of caspases (44, 45). Caspase-3-induced cleavage of PKCδ separates the PKC catalytic domain from the regulatory (inhibitory) domain. Consequently, caspase-dependent PKCδ cleavage results in increased PKC activation (46). Significantly, MPP+ was reported to induce proteolytic cleavage and activation of the PKCδ isoform through activation of caspase-3 (41). To determine whether PKCδ mediated MPP+ effects on FAT, we coperfused squid axoplasm with MPP+ and PKCδ peptide substrate (PKCδS), a highly specific peptide substrate for PKCδ corresponding to residues 422–443 of murine elongation factor eEF-1α (47). *In vitro* kinase assays show PKCδS is an absolutely specific substrate for PKCδ, and a structural basis

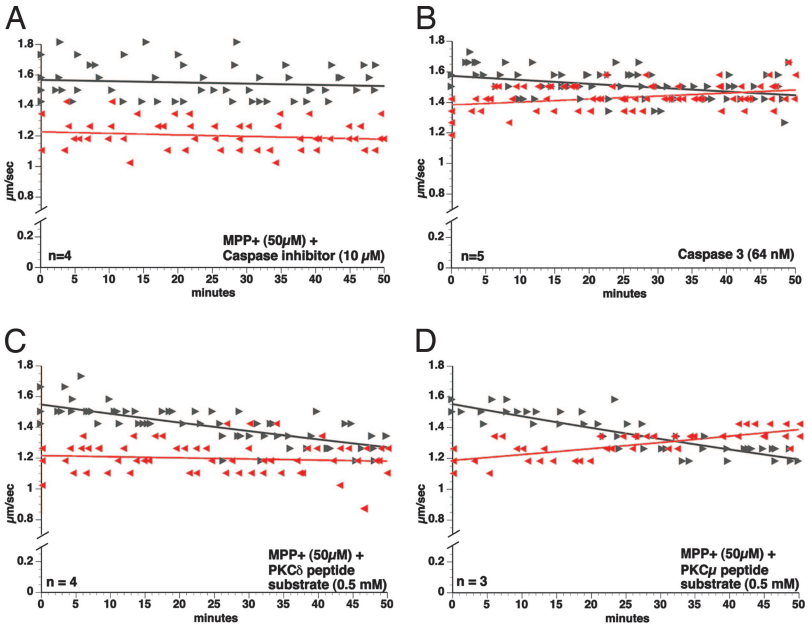

Fig. 4. Both a caspase inhibitor and a PKCδ substrate block the effects of MPP+ on FAT. (*A*) Coperfusion of MPP+ with the caspases-3 inhibitor, caspase inhibitor II, blocks the effects of MPP+ on FAT. (*B*) Consistent with the proposal that caspase-mediated cleavage of PKCδ mediates MPP+ effects on FAT, perfusion of axoplasm with low levels of active caspase-3 activates retrograde FAT and inhibits anterograde FAT. (*C*) Coperfusion of MPP+ with a peptide substrate highly specific for PKCδ protects CDyn-mediated FAT. (*D*) In contrast, coperfusion of MPP+ with a peptide substrate highly specific for PKCμ fails to protect FAT, suggesting that PKCδ mediates actions of MPP+ and caspase.

of PKCδ selectivity toward PKCδS was shown (47). Coperfusion of MPP+ with PKCδS blocked MPP+-induced activation of CDyn-based motility (Fig. 4*C*). In contrast, coperfusion of MPP+ with a specific peptide substrate for the PKCμ isoform failed to block MPP+ effects on FAT (Fig. 4*D*). Taken together, these results indicated that caspase-dependent activation of PKCδ mediates MPP+ effects on FAT.

MPP+ Treatment Dramatically Reduces the Number of Membrane Profiles in Presynaptic Terminals. Results presented here show that MPP+ affected FAT by activation of endogenous caspase and PKCδ enzymatic activities. One predicted consequence of MPP+ effects on FAT is the depletion of membrane profiles from distal axonal compartments. Presynaptic terminals are well defined distal axonal compartments, which require a regulated flow of membrane trafficking for their proper function. To evaluate the functional consequences of MPP+ treatment of distal axons, we examined the presynaptic compartment of MPP+ and control-injected squid giant synapses by electron microscopy (Fig. 5). Remarkably, ultrastructural analysis of MPP+-injected synapses revealed a dramatic reduction in the number of membrane profiles and synaptic vesicles (Fig. 5*B*) compared with control buffer-injected ones (Fig. 5*A*). These changes were consistent with depletion of the distal axon by enhanced retrograde FAT and raised the possibility of altered synaptic function as a consequence of MPP+-included alterations in vesicle trafficking. A detailed description of the effects of MPP+ injection on both synaptic transmission and ultrastructure are provided in the accompanying paper by Serulle *et al.* (48). Taken together, these results suggest that increased CDyn-dependent motility induced by MPP+ has a direct impact on the number of membranous organelles in presynaptic terminals.

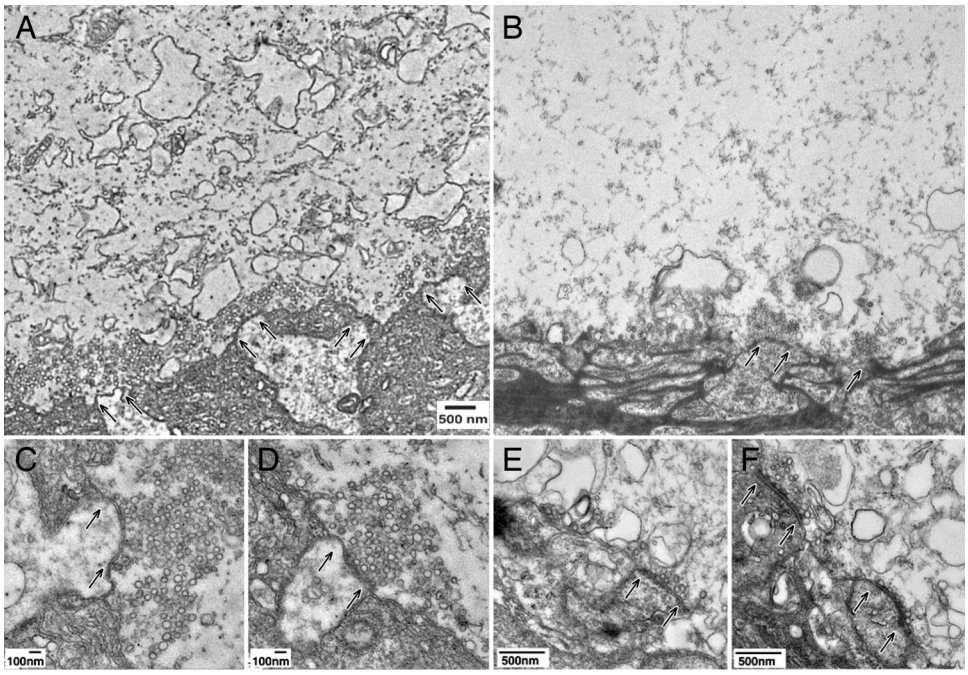

Fig. 5. MPP+ depletes membranous profiles from the presynaptic terminal. Electron micrographs of control-injected (*A*, *C*, and *D*) and MPP+ injected (*B*, *E*, and *F*) presynaptic terminals of the squid giant synapse. In low magnification images [compare control (*A*) to MPP+ (*B*)], the amount of membranous profiles are substantially reduced with MPP+ treatment. At higher magnification, regions near active zones have abundant vesicles present in control injected synapses (*C* and *D*), but few vesicles remain near active zones (*E* and *F*) after MPP+ injections. Arrows denote synaptic active zones.

Discussion

Understanding pathogenic mechanisms for adult-onset neurological diseases represents a major challenge. A large body of experimental evidence has accumulated that neuronal cells follow a typical dying-back pattern of degeneration in most of these diseases. Dying-back neuropathies are characterized by a sequence that progresses from loss of synaptic function to distal axonopathy and eventually to neuronal death (49, 50). Supporting this idea, presymptomatic alterations in synaptic function have been documented in human and animal models of Parkinson's, Alzheimer's, and Huntington's diseases as well as Amyotrophic Lateral Sclerosis (51).

Although molecular mechanisms leading to this sequence of pathogenic events in adult-onset neurological diseases have remained unclear, disruptions in FAT suffice to produce this pattern (18). For example, several dying-back neurodegenerative diseases have been linked to loss-of-function mutations in MT-based molecular motors responsible for FAT, including kinesin-1, and selected CDyn subunits (19). These findings highlighted the requirement of maintaining appropriate levels of anterograde and retrograde FAT for neuronal function and survival (10). However, mutations in motor proteins are rare and have not been linked with adult-onset neurological diseases like Alzheimer's, Parkinson's and PolyQ-expansion diseases. Alternatively, recent evidence suggests that misregulation of FAT through altered kinase activities may compromise FAT in Huntington's (31), Kennedy's (30, 31), and Alzheimer's disease (52, 53).

Pathological observations derived from both toxin-induced and sporadic PD cases indicate that PD is a dying-back neuropathy (20). Specifically, loss of striatal presynaptic terminals precedes loss of neuronal cell bodies in the substantia nigra pars compacta and is more extensive when clinical symptoms develop (22, 23). Evidence of early axonal dysfunction in PD (54) prompted us to examine axon-specific events in a toxin-induced PD model. MPP+ is a well established model of PD (3), but the effect of this compound on isolated axonal and presynaptic compartments had not been examined.

Squid axoplasm vesicle motility assays and giant synapse injection experiments evaluated effects of MPP+ on FAT and synaptic transmission, respectively. The squid axoplasm preparation represents a well characterized system for study of molecular motors (55, 56), FAT regulation (28, 33, 52), and pathogenic mechanisms underlying dying-back neuropathies (10, 30). This model allowed us to evaluate MPP+ effects at defined concentrations, independent of effects on cell body-related activities (i.e., transcriptional changes).

Perfusion of MPP+ in squid axoplasm induced rapid and dramatic changes in FAT. Specifically, MPP+ induced a decrease in kinesin-dependent anterograde FAT rates while increasing CDyn-based retrograde FAT. MPP+ has been reported to block mitochondrial ATP production by inhibiting complex I (32), but this does not explain observed effects on FAT. Uncouplers of oxidative phosphorylation inhibit both directions of FAT in the absence of exogenous ATP (26). However, MPP+ induces an increase in CDyn-dependent motility, which depends on ATP hydrolysis and exogenous ATP was provided. Although mitochondria may play a role in the activation of caspase 3 by MPP+, the changes observed here do not reflect reduced cellular ATP levels.

In vivo, FAT is regulated by phosphorylation-dependent mechanisms (10, 33). Multiple kinase pathways have been identified that affect FAT through phosphorylation of specific molecular motor subunits (28, 30, 52). Significantly, numerous reports indicated that MPP+ treatment of cells results in alterations in the activity of several protein kinases (41, 57–59) and abnormal patterns of protein phosphorylation (60, 61). This suggested that axonal kinases might mediate MPP+ effects on FAT.

Squid axoplasm experiments showed that perfusion of PKC catalytic subunit elicited effects similar to those induced by MPP+ (33), implicating PKC kinases as mediators of MPP+ effects on FAT. Coperfusion of MPP+ with pharmacological inhibitors of PKC blocked its effects on FAT, consistent with this idea. However, these experiments did not identify specific PKC isoforms involved. Previous studies ruled out pathways mediated by conventional, calcium- and DAG-activated PKCs (PKCα, β, and γ) (62). The absence of a plasma membrane in the isolated axoplasm model similarly eliminates the possibility that membrane receptor-mediated signaling pathways are required for activation of PKC. To identify PKC isoforms activated by MPP+ in axoplasm, we coperfused axoplasm with MPP+ and isoform-specific PKC peptide substrates of selected novel PKCs. A peptide substrate highly specific for the novel PKCδ completely blocked effects of MPP+ on FAT, but a peptide substrate for PKCμ did not. Significantly, previous studies reported activation of PKCδ in MPP+-treated cells (41). Interestingly, alterations in PKC activity were reported recently to underlie synaptic dysfunction in spinocerebellar ataxia (63) and viral-induced behavioral changes (64).

Previous studies suggested that PKCδ activation by MPP+ was mediated by proteolytic cleavage of PKCδ by caspase-3 (41). Consistent with this, coperfusion of MPP+ and Z-DEVD-fmk, a peptide inhibitor of caspase 3, also blocked effects of MPP+ on FAT. Protective actions of Z-DEVD-fmk suggested that MPP+ treatment results in axonal and presynaptic activation of caspase-3, leading to local cleavage of PKCδ and local regulation of membrane-bound organelle trafficking. Mechanisms underlying MPP+-induced caspase activation are not addressed in these studies, but a role for mitochondria in caspase activation is well established (65, 66). Our observations raise the possibility of novel, neuronal-specific actions for caspase activation in axons and presynaptic terminals.

As noted in the accompanying article (48), MPP+ treatment leads to failure of synaptic transmission by a mechanism that involves removal of synaptic vesicles from presynaptic terminals. Changes in FAT elicited by MPP+ would affect availability of membrane components essential for maintenance of synaptic function. Both FAT and synaptic effects are prevented by inhibitors of PKCδ and caspases.

Together, these observations suggest that PD and other CNS pathologies associated with a loss of synaptic function characteristic of a dying-back neuropathy may result from deregulation of FAT. We propose the term "dysferopathy" (from the Greek "fero," meaning to carry or to transport) to describe pathologies associated with FAT deregulation. The hallmark of a dysferopathy would be compromises in FAT that lead to a late-onset, dying-back neuropathy. Misregulation of FAT would reduce availability of synaptic vesicles and other presynaptic membrane-associated components required for the maintenance of synaptic function, thus providing an explanation for both synaptic dysfunction and the dying-back neuropathy phenotype.

Materials and Methods

Reagents. CREBpp was from New England Biolabs. Peptide substrates for PKCδ (PKCδS, 539563) and PKCμ (539564), MARCKS (454880), PKC catalytic subunit (539513), Gö6976 (369513), Gö6983 (365251), and caspase inhibitor II (Z-DEVD-fmk, 264155) were from Calbiochem (San Diego, CA). MPP+ (D048) was from Sigma. Active recombinant caspase 3 (14-264) was from Upstate Biologicals (Lake Placid, NY).

Motility Studies in Axoplasm. Axoplasm was extruded from giant axons of the squid Loligo pealeii at Marine Biological Laboratory and perfused as described in refs. 28 and 30.

PKC Activity in Control and MPP+-Treated Axoplasm. Extruded axoplasms on coverslips were incubated without (control) or with

200 μM MPP+ in buffer X/2 (26, 30) containing 100 μM ATP for 45 min at room temperature. After incubation each was transferred to a 1.5-ml tube containing 100 μl of 1/80 protease inhibitor mixture (P8340; Sigma), 3 mM EDTA, and 1 μM protein kinase A inhibitor (NEB). Samples were triturated with P200 Gilson pipettor followed by 5 strokes with a 250-μl Hamilton syringe and a 27-gauge needle. Samples were clarified by centrifugation at 16,000 $\times$ *g* at room temperature for 5 min. Supernatants were transferred to new 1.5-ml tubes and kept at 4°C. Kinase reactions were started by adding 17 μl of control or MPP+ clarified supernatants to 6 μl of 5$\times$ kinase buffer (250 mM K aspartate/50 mM $MgCl_2$/5 mM DTT/100 mM Hepes, pH 7.4/500 μM cold ATP/500 nM okadaic acid/1.5 mCi [γ-^{32}P]ATP; 1 Ci = 37 GBq) brought to a final volume of 30 μl with 20 mM Hepes, pH 7.4. Three parallel assays were run: buffer control for axoplasmic protein phosphorylation, 50 μM MARCKS for PKC activity, and 200 μM CREBpp as an internal standard. Reactions were stopped by addition of 100 μl of 10% trichloroacetic acid (TCA). TCA supernatants were spotted on P81 phosphocellulose circles, washed three times in 75 mM phosphoric acid, dried, and analyzed by scintillation counting. PKC activity in axoplasm is expressed as the ratio of radioactivity on MARCKS to CREBpp peptide.

Electron Microscopy of Presynaptic Terminals. Electron microscopy was performed as described in ref. 67. Briefly, ganglia were fixed by immersion in 6% glutaraldehyde in Ca^{2+}-free seawater, postfixed in osmium tetroxide, and in-block-stained with uranyl acetate. Fixed ganglia were dehydrated in ethanol, substituted with propylene oxide, and embedded in Araldite resin (CY212) or Embed 812 (EM Science). Ultrathin sections were collected on Pyoloform (Ted Pella, Redding, CA) and carbon-coated single-slot grids, contrasted with uranyl acetate and lead citrate, observed, and digitally photographed in a JEOL 200CX transmission electron microscope adapted with an AMT digital camera or scanned negatives taken with a Philips 208 transmission electron microscope. Electron micrographs were taken at initial magnifications of $\times$10,000–$\times$30,000.

Statistical Analysis. All experiments were repeated at least three times. Unless otherwise stated, the data were analyzed either by an analysis of variance (ANOVA) followed by post hoc Student–Newman–Keul's test to make all possible comparisons or by a pooled *t* test of μ1-μ2. Data were expressed as mean $\pm$ SEM, and significance was assessed at $P < 0.05$ or $P < 0.01$ as noted.

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